# Different membrane targeting of prostaglandin EP3 receptor isoforms dependent on their carboxy-terminal tail structures

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**Abstract Mouse prostaglandin EP3 receptor consists of three isoforms, EP3α, β and γ, with different carboxy-terminal tails. To assess the role of their carboxy-terminal tails in membrane targeting, we examined subcellular localization of myc-tagged EP3 isoforms expressed in MDCK cells. Two isoforms, EP3α and EP3β, were localized in the intracellular compartment but not in the plasma membrane, while the EP3γ isoform was found in the lateral plasma membrane and in part in the intracellular compartment. Mutant EP3 receptor lacking the carboxy-terminal tail was localized in the intracellular compartment but not in the plasma membrane. Thus, EP3 isoforms differ in subcellular targeting, and the carboxy-terminal tails play an important role in determination of the membrane targeting of EP3 receptor.**



*Key words:* Prostaglandin; G protein-coupled receptor; Targeting; MDCK cell

## 1. Introduction

Prostaglandin (PG) $E_2$ exhibits a broad range of biological actions in diverse tissues through its binding to specific receptors on plasma membrane [1]. PGE receptors are pharmacologically subdivided into four subtypes, EP1, EP2, EP3, and EP4, on the basis of their responses to various agonists and antagonists [2,3]. Among the four subtypes, the EP3 subtype has been most well characterized and involved in diverse $PGE_2$ actions [3]. We have cloned the mouse EP3 receptor and demonstrated that this receptor is a G protein-coupled receptor (GPCR) that engages in inhibition of adenylate cyclase [4]. Furthermore, we have identified the three isoforms of the mouse EP3 receptor with different carboxy-terminal tail, EP3α, EP3β and EP3γ, which are produced through alternative splicing [5,6]. Our previous studies revealed that these isoforms differed in agonist dependent desensitization [7] and in agonist-independent constitutive Gi activity [8,9].

One of the prominent EP3 actions is inhibition of sodium and water reabsorption in collecting tubules of kidney [10], and this action is mediated by Gi [11]. Like all other epithelial cells, renal tubule cells are highly polarized, and their plasma membranes are divided into two separate membrane domains, apical and basolateral [12]. Many membrane proteins tend to be localized in either the apical or the basolateral membrane, and the polarized distribution is thought to be important for the function of these proteins. Although either luminal or basolateral $PGE_2$ has been reported to exhibit Gi activity in canine cortical collecting tubule cells [13], membrane polarization of EP3 receptors has not yet been elucidated. We here investigated subcellular targeting of three EP3 isoforms in Madin–Darby canine kidney (MDCK) cells, which are generally used as a model of polarized epithelial cells, and demonstrated that these isoforms differed in subcellular targeting, that is, EP3α and EP3β isoforms were localized in the intracellular compartment, while EP3γ isoform was targeted to the lateral plasma membrane.

## 2. Materials and methods

### 2.1. Materials

MDCK cell line, sulprostone and anti-telencephalin (tln) antibody were generous gifts from Drs. K.E. Mostov (University of California, San Francisco, CA, USA), K.-H. Thierauch (Schering, Germany) and Y. Yoshihara (RIKEN Brain Science Institute, Saitama, Japan), respectively. cDNA for rabbit tln was also kindly provided by Dr. Y. Yoshihara. Alexa™488-phalloidin was purchased from Molecular Probes; rhodamine-conjugated goat $F(ab')_2$ anti-rabbit IgG (H and L) and rhodamine-conjugated goat $F(ab')_2$ anti-mouse IgG (H and L) were from Leinco Technologies.

### 2.2. DNA construction

Mutant EP3 receptor T-335, which lacked the C-terminal domain from the alternative splicing site, was generated as described previously [14]. For construction of myc-tagged receptors, cDNAs of three EP3 isoforms and T-335 receptor were cloned in frame into a pcDNA3 eukaryotic expression vector (Invitrogen) containing the myc-epitope tag sequence at the 5′ end. C-terminal-truncation mutant tln (tln-S), lacking the last 56 amino acid residues, was generated by PCR-mediated mutagenesis, and subcloned into a pcDNA3 vector. For chimeric mutants of tln with the last 30 amino acid residues of the mouse EP3α (tln-α) or the last 29 amino acid residues of the mouse EP3γ (tln-γ) instead of the C-terminal domain of tln, a *Kpn*I site was created after the last amino acid of tln-S by PCR-mediated mutagenesis, and this construct was subcloned into the pBluescript SK(+) (tln-K/pBs). *Kpn*I/*Eco*RI or *Kpn*I/*Xba*I fragment encoding the C-terminal domain of mouse EP3α or EP3γ isoform was generated by PCR-mediated mutagenesis and fused with the *Kpn*I site of tln-K/pBs. After complete sequencing, these fusion cDNAs were subcloned into a pcDNA3 vector.

### 2.3. Cell culture and transfection

MDCK cells were maintained as described previously [15]. To establish the MDCK cell lines, stably expressing myc-tagged EP3 isoforms, MDCK cells were transfected with their cDNAs by CellPhect Transfection kit (Pharmacia), and stable transformants were cloned by selection with G-418 (Gibco BRL). For transient expression, MDCK cells were transfected with each cDNA by Lipofectamine 2000 reagent (Gibco BRL) for 6 h in Opti-MEM (Gibco BRL), fol-

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*Abbreviations:* PG, prostaglandin; GPCR, G protein-coupled receptor; MDCK, Madin–Darby canine kidney; tln, telencephalin


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lowed by addition of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and incubated for 3 days.

*2.4. Immunofluorescence*

MDCK cells were seeded onto poly-L-lysine-coated glass coverslips in 12-well plates. For localization of EP3 isoforms or tln mutants, immunofluorescence microscopy was performed. The cells were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 3% sucrose for 30 min at 4°C. Cells were then permeabilized with TPBS/HS (10 mM phosphate buffer containing 0.1 M NaCl and 0.1% Tween 20), containing 0.1% Triton X-100 at room temperature for 10 min and washed with TPBS/HS twice. They were blocked with 0.5% bovine serum albumin in TPBS/LS (phosphate-buffered saline containing 0.05% Tween 20) for 1 h at room temperature and rinsed with TPBS/HS. To identify the EP3 receptors or tln mutants, cells were incubated with antibody against myc tag (9E10) (2 μg/ml) or tln (1:10 dilution) in TPBS/HS at 4°C overnight. They were then washed with TPBS/HS three times and incubated with rhodamine-conjugated secondary antibody (1:200 dilution) at room temperature for 1 h. F-actin was visualized with Alexa™488-phalloidin (1:400 dilution), and used as a marker of the plasma membrane, as described previously [16]. They were washed with TPBS/HS three times and mounted onto a slide glass in phosphate-buffered saline/glycerol containing *p*-phenylenediamine dihydrochloride. Cells were photographed under a laser scanning confocal microscope (Bio-Rad, MRC-1024).

## 3. Results

*3.1. Membrane targeting of three EP3 isoforms in MDCK cells*

To examine the membrane targeting of the three EP3 isoforms, we transiently expressed the myc-tagged isoforms in MDCK cells. To avoid the effect of endogenously produced $PGE_2$ on the receptor localization, the cells were pretreated with indomethacin, a cyclooxygenase inhibitor, before staining. In the cells expressing EP3α and EP3β isoforms, the abundant myc staining was present in the intracellular, perinuclear compartment (Fig. 1A,B), while the staining was observed in neither the apical nor the basolateral plasma membrane (Fig. 1E,F). On the other hand, although EP3γ isoform was in part localized in the intracellular compartment as observed in EP3α- and EP3β-expressing cells, this isoform was accumulated in the lateral plasma membrane but not in the apical and basal plasma membranes (Fig. 1C,G). To assess the role of the C-terminal tails in the membrane targeting, we next tried to investigate the subcellular localization of T-335, C-terminal tail-truncation mutant receptor. Similar to EP3α and β isoforms, transiently expressed myc-tagged T-335 was localized in the intracellular, perinuclear compartment, and no staining was seen in the plasma membrane (Fig. 1D,H).

To exclude aberrant localization due to transient overexpression of the isoforms, we established the MDCK cell lines stably expressing the myc-tagged mouse EP3α, β, or γ isoform. Scatchard analyses of $PGE_2$ binding to the MDCK cell membranes expressing each isoform showed that these isoforms displayed high affinities for $PGE_2$ with dissociation constants of 4.9 nM for EP3α, 8.1 nM for EP3β and 19.6 nM for EP3γ isoforms (data not shown). These values are comparable to those obtained with CHO cells expressing each isoform [5,6]. EP3α and EP3β isoforms were localized in the intracellular, perinuclear region, and there was not enrichment of both isoforms in the plasma membrane (Fig. 2A,B,D,E). On the other hand, EP3γ isoform was mainly found in the lateral plasma membrane, though the isoform was in part localized in the intracellular compartment (Fig. 2C,F). These results are well consistent with those obtained by the transient expression system.

These results taken together demonstrate that three EP3 isoforms show different subcellular targeting in MDCK cells, indicating that the C-terminal tails play a crucial role for the determination of subcellular targeting of EP3 receptors.

We next examined the effect of agonist treatment on the localization of EP3 isoforms. Whereas sulprostone, an EP3 agonist, did not affect the localization of EP3α and EP3β isoforms (Fig. 3A,B,D,E), EP3γ isoform in the lateral plasma

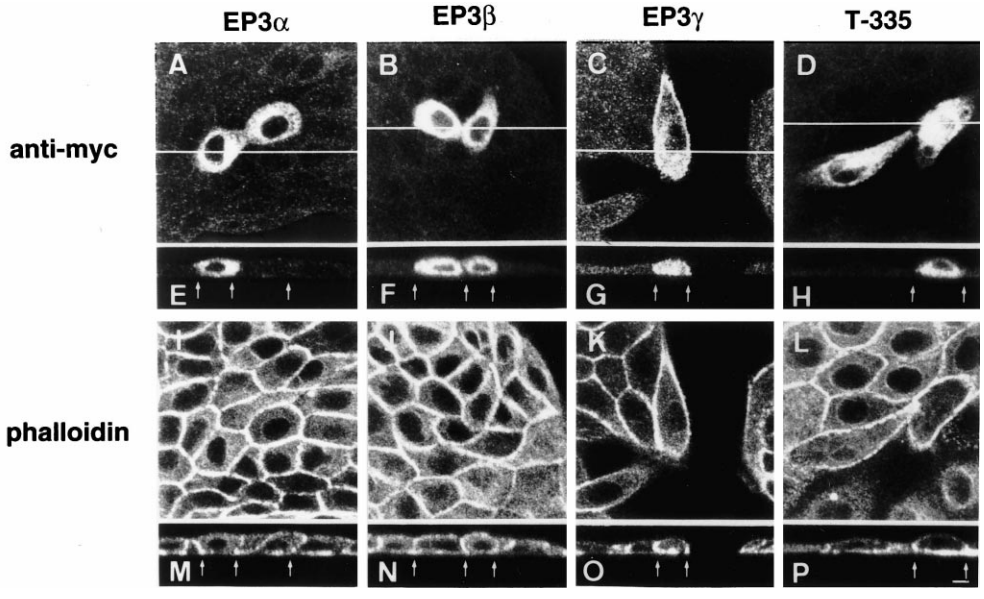


Fig. 1. Transient expression of three EP3 isoforms and T-335 in MDCK cells. After MDCK cells transiently transfected with EP3α (A, E, I and M), EP3β (B, F, J and N), EP3γ (C, G, K and O) or T-335 (D, H, L and P) had been treated with 10 μM indomethacin in serum-free medium for 2 h, they were fixed and stained with anti-myc antibody (A–H) and Alexa™488-phalloidin (I–P). They were analyzed by confocal microscopy (A–D and I–L, *x-y* images; E–H and M–P, *x-z* images). The white line represents the section of cells through which the *z* scan was made. The arrows indicate the lateral membranes. The results shown are representative of three independent experiments that yielded similar results. The bar represents 10 μm.

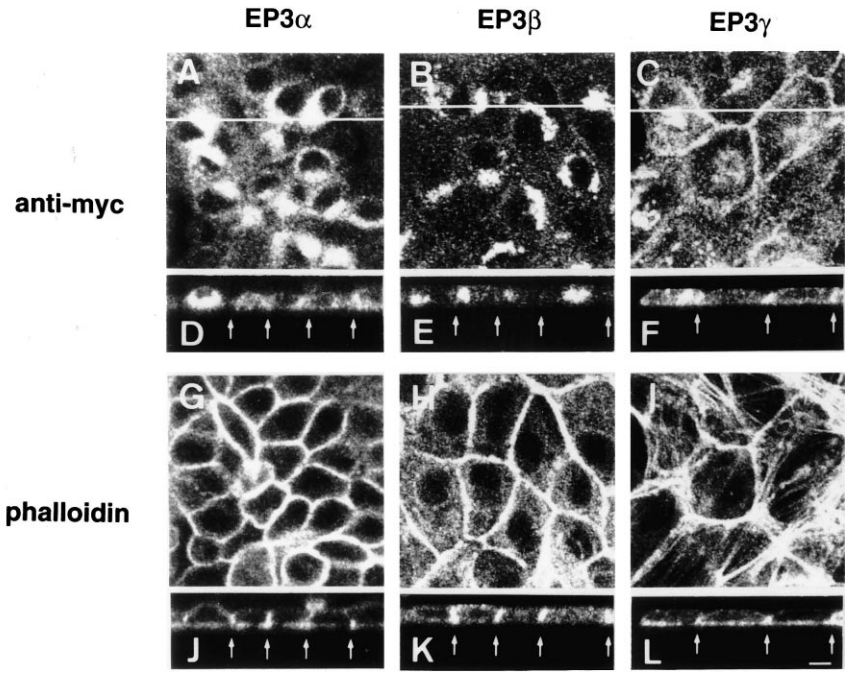


Fig. 2. Stable expression of three EP3 isoforms in MDCK cells. After MDCK cells stably expressing EP3α (A, D, G and J), EP3β (B, E, H and K) or EP3γ (C, F, I and L) had been treated with 10 μM indomethacin in serum-free medium for 2 h, they were fixed and stained with anti-myc antibody (A–F) and Alexa™488-phalloidin (G–L). They were analyzed by confocal microscopy (A–C and G–I, *x-y* images; D–F and J–L, *x-z* images). The white line represents the section of cells through which the *z* scan was made. The arrows indicate the lateral membranes. The results shown are representative of three independent experiments that yielded similar results. The bar represents 10 μm.

membrane disappeared by the incubation with sulprostone (Fig. 3C,F), suggesting that EP3γ isoform in the lateral plasma membrane was internalized.

### 3.2. *The effects of the C-terminal tails of EP3 isoforms on the subcellular localization of tln*

Tln is a cell adhesion molecule which is distributed in telencephalic neurons [17,18]. In order to examine membrane targeting activity of the C-terminal tails of EP3 isoforms, we constructed the cDNAs for the cytoplasmic domain-truncated mutant tln (henceforth referred to as 'tln-S') and tln harboring the C-terminal tail of EP3α or EP3γ isoform instead of the cytoplasmic domain of tln (referred to as 'tln-α' or 'tln-γ') (Fig. 4). When wild type tln was transiently expressed in MDCK cells and stained with anti-tln antibody, wild type tln was localized mainly in the apical plasma membrane and additionally in the lateral plasma membrane (Fig. 4A,E). No staining was observed in the intracellular compartment, cytoplasm, and the basal plasma membrane. On the other hand, tln-S lost the apical membrane targeting activity but was re-

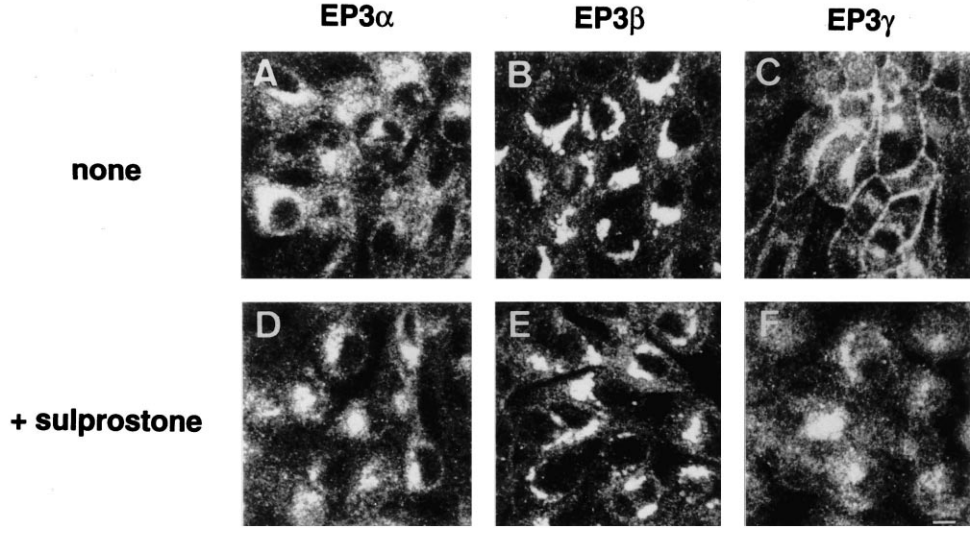


Fig. 3. Effect of sulprostone on the subcellular localization of the EP3 isoforms. After MDCK cells stably expressing EP3α (A and D), EP3β (B and E) or EP3γ (C and F) had been treated with 10 μM indomethacin in serum-free medium for 1 h, they were incubated with (D–F) or without (A–C) 1 μM sulprostone for 1 h. They were then fixed and stained with anti-myc antibody. They were analyzed by confocal microscopy. The results shown are representative of three independent experiments that yielded similar results. The bar represents 10 μm.

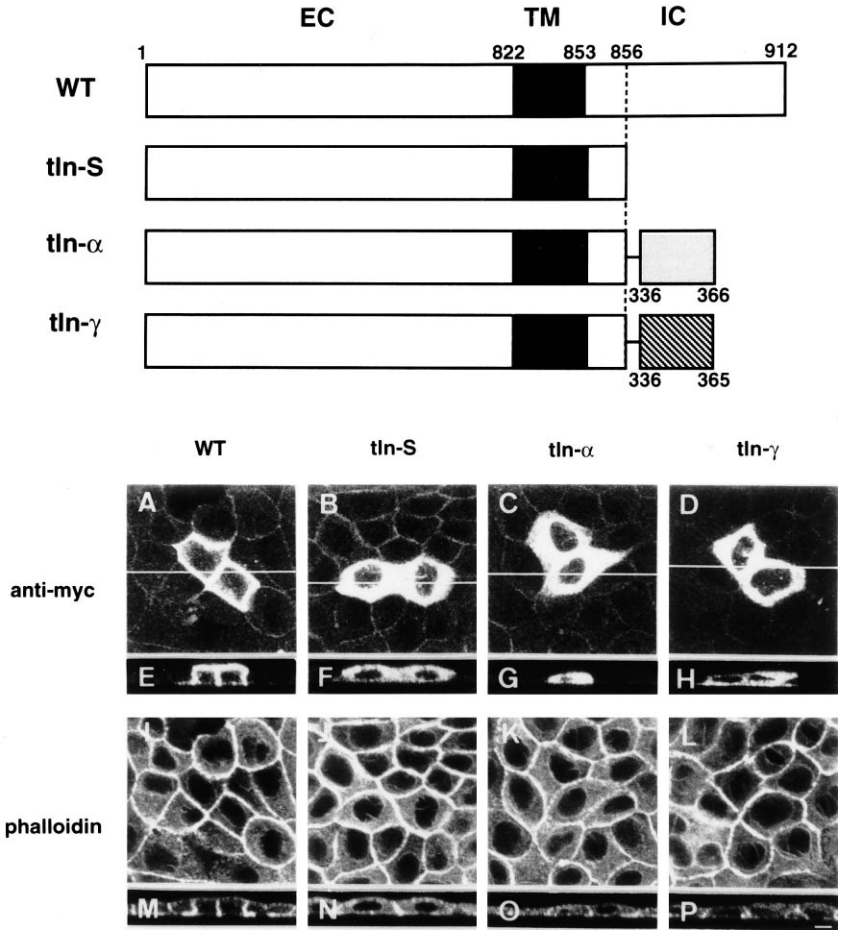


Fig. 4. Expression of tln mutants in MDCK cells. Upper panel: Schematic representation of constructed tln mutants. Open boxes represent the sequence of tln. Transmembrane domain of tln is shown by closed boxes. Shaded box and stripped box indicate the C-terminal tail of EP3α and EP3γ isoforms, respectively. The numbers shown above and below the boxes indicate the amino acid numbers of tln and EP3 isoforms, respectively. EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain. Lower panel: After MDCK cells transiently transfected with wild type tln (A, E, I and M), tln-S (B, F, J and N), tln-α (C, G, K and O) or tln-γ (D, H, L and P) had been serum-starved for 2 h, they were fixed and stained with anti-tln antibody (A–H) and Alexa™488-phalloidin (I–P). They were analyzed by confocal microscopy (A–D and I–L, *x-y* images; E–H and M–P, *x-z* images). The white line represents the section of cells through which the *z* scan was made. The results shown are representative of three independent experiments that yielded similar results. The bar represents 10 μm.

tained in the intracellular compartment (Fig. 4B,F), indicating that the cytoplasmic domain of tln is required for the apical membrane targeting. Meanwhile tln-α and tln-γ were localized in the intracellular compartment but not in the plasma membrane (Fig. 4C,D,G,H).

## 4. Discussion

We have examined membrane targeting activities of three EP3 isoforms and presented here that EP3α and β isoforms were retained in the intracellular compartment while EP3γ isoform was targeted to the lateral plasma membrane in MDCK cells. EP3α and β isoforms showed high affinity $PGE_2$ binding, demonstrating that both isoforms appear to be functional in the intracellular compartment. In agreement with this finding, the human EP3α isoform was recently reported to be localized in the nuclear envelope and could affect transcription of genes and intranuclear calcium transients [19]. Recent studies have revealed intracellular compartment distribution of GPCRs, such as thrombin receptor and dopamine receptor [20,21]. When the C-terminal tail was truncated from the EP3 receptor, the mutant receptor without the C-terminal tail was retained in the intracellular compartment, suggesting that the C-terminal tails of EP3α and β isoforms have no ability to recruit the receptor to the plasma membranes.

In contrast to EP3α and β isoforms, EP3γ isoform was targeted to the lateral plasma membrane in MDCK cells. Considering localization of C-terminal tail-truncated mutant T-335 to the intracellular compartment, the C-terminal tail of EP3γ isoform is a determinant for targeting to the lateral plasma membrane. The domains of membrane targeting of GPCRs have been extensively studied, and the C-terminal domain has been shown to play an important role in the targeting of several GPCRs. Two subtypes of metabotropic glutamate receptor, mGluR2 and mGluR7, have been reported to be targeted to the somatodendritic and axonal membranes, respectively, in cultured hippocampal neurons, their targeting specificities being determined by the sequence of the C-terminal tail [22]. Besides, Wong et al. reported that two isoforms of monocyte chemoattractant protein-1 receptor (CCR2A and CCR2B), which were produced by alternative splicing and differed in the C-terminal sequence, were distributed at the cytoplasm and in the plasma membrane, respectively, in HEK293 cells [23]. To assess the membrane targeting

activity of the C-terminal tail of EP3γ isoform, we constructed the unrelated apical protein tln harboring the C-terminal tail of EP3γ isoform instead of the cytoplasmic domain of tln, which acts as the targeting signal of tln to the apical membrane. However, the C-terminal tail of EP3γ isoform failed to recruit tln to the plasma membrane, indicating that the C-terminal tail of EP3γ isoform is not sufficient for the plasma membrane targeting. Limbird and co-workers have shown multiple determinants, including the third intracellular loop, for the basolateral membrane localization of $\alpha_{2A}$-adrenergic receptor in MDCK cells [24,25]. In the light of these observations, although the C-terminal tail of EP3γ isoform is an important determinant for the lateral membrane targeting, another domain(s) of the receptor appears to be needed for the targeting.

We here showed that three EP3 isoforms differed in membrane targeting in MDCK cells and the C-terminal tails were crucial determinants for membrane targeting. Thus, alternative splicing creates three EP3 isoforms with distinct properties at the level of subcellular targeting. In cells, especially polarized cells, different subcellular targeting of EP3 receptor isoforms would provide distinct cellular functions of the receptor dependent on targeted sites, even if the isoforms are coupled to the same signal transduction pathway. This finding will be of help in understanding the diversity of cellular responses to $PGE_2$.

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